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Constitutively Elevated Levels of Putrescine and Putrescine-Generating Enzymes Correlated with Oxidant Stress Resistance in *Conyza bonariensis* and Wheat[1,2]

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Oxidant stress resistance in *Conyza bonariensis* and wheat (*Triticum aestivum*) has been correlated with high levels of antioxidant enzyme activities. Additionally, external oxidant stresses can increase a plant's levels of the enzymes of polyamine biosynthesis and polyamines, especially putrescine. We investigated the constitutive relationships between putrescine, putrescine-generating enzymes, and oxidant stress resistance in wheat and *C. bonariensis*. Putrescine was constitutively elevated (2.5- to 5.7-fold) in 2-week-old-resistant wheat and *C. bonariensis* biotypes, which correlated with a 10- to 15-fold increase in paraquat oxidant resistance. Arginine and ornithine decarboxylase activities doubled, along with higher putrescine levels in resistant *C. bonariensis*. The variations in levels of putrescine and arginine and ornithine decarboxylase activities paralleled the constitutive variation of antioxidant enzymes, as well as oxidant resistance. Higher levels of both putrescine and antioxidant enzyme activities occurred during a peak of oxidant resistance at 10 weeks, when paraquat resistance in *C. bonariensis* plants is >50-fold greater than in the sensitive biotype. Application of 100 μM putrescine can double oxidant-stress resistance in the resistant *C. bonariensis*. Putrescine may play an important role in contributing to the base level of oxidant resistance found at the nonpeak period.

Polyamines are present in cells at up to millimolar amounts and are considered to be important for DNA replication, cell differentiation, and growth regulation (Galston and Sawhney, 1990). Of these, putrescine levels and biosynthesis often change in response to different environmental stresses in plants, e.g. mineral deficiencies (Basso and Smith, 1974), low pH (Young and Galston, 1983), osmotic stress (Flores and Galston, 1984; Tiburcio et al., 1986; Aziz and Larher, 1996), sulfur dioxide (Preibe et al., 1978), ozone (Langebartels et al., 1991), and UV-B radiation (Kramer et al., 1991). Polyamines can act directly as free radical scavengers (Drolet et al., 1986), or function as scavengers by interacting with other molecules such as free ferulic and caffeic acids (Bors et al., 1989). Leaf necrosis caused by ozone in tomato plants could be prevented by exogenously supplying putrescine, spermidine, and spermine (Ormrod and Beckerson, 1986).

Plant cells can be protected against oxidant stress by various radical-scavenging systems, including low-molecular-weight antioxidants such as ascorbate, glutathione, α-tocopherol, and carotenoids, as well as by antioxidant enzymes such as superoxide dismutases, peroxidases, and glutathione reductases (Foyer et al., 1994). The antioxidant enzyme activities are constitutively higher in the resistant biotypes than in the sensitive biotypes of many species (Gressel and Galun, 1994). Higher levels of polyamines correlated with paraquat resistance in *Conyza canadensis* (Szigeti et al., 1996). We tested whether this might be so in two other systems: (a) An oxidant-resistant biotype of the closely related *Conyza bonariensis*, which has developmentally controlled levels of oxidant resistance in which only the highest level of resistance correlated with increased antioxidant enzymes (Amsellem et al., 1993; Ye and Gressel, 1994) and the antioxidant enzyme activities and levels of mRNAs coding for the antioxidant enzymes can be induced to higher levels by oxidant stress pretreatment (but only in the resistant *C. bonariensis* biotype and not in the sensitive biotype). (b) A drought-tolerant wheat (*Triticum aestivum* L.) line with high tolerance to photooxidant stress, which was correlated with higher levels of glutathione reductase and ascorbate peroxidase activities (Pastori and Trippi, 1993). Different levels of resistance against drought were correlated with resistance to high-oxygen tension in cv Cruz Alta (stress-tolerant), whereas cv Leones was stress-sensitive. The resistant line could be induced to higher levels of oxidant tolerance by oxidant stress, and this correlated with higher levels of these enzymes.

In this report we confirm and extend the findings of Szigeti et al. (1996) by showing that putrescine levels are also constitutively elevated in a drought-resistant wheat variety and in an oxidant-stress-resistant *C. bonariensis* biotype. We further extended their results by finding that constitutively elevated putrescine levels were correlated with elevated levels of putrescine-generating enzymes: Arg decarboxylase (EC 4.1.1.19) and Orn decarboxylase (EC 4.1.1.17). Their activities are specifically elevated in the

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Abbreviation: I_{50}, concentration necessary to achieve 50% inhibition.

resistant plants. We also show that the levels of each enzyme vary during plant development. In addition, we show that the level of paraquat-induced oxidant resistance can be further enhanced by exogenous application of putrescine. These findings further support the contention that putrescine has an antioxidant defense function in plants.

MATERIALS AND METHODS

Two biotypes of *Conyza bonariensis* (L.) Cronq. and two genotypes of wheat (*Triticum aestivum* L.), having well-defined differences in oxidant-stress resistance, were chosen for these experiments. The seeds of *C. bonariensis* were originally gathered in Egypt, the wild type near Alexandria and the resistant biotype from the Tahrir irrigation district, where paraquat resistance evolved. The *C. bonariensis* seeds were germinated and grown in a culture room with a 10-h light period of 100 μmol m^{-2} s^{-1} PPFD at 25 ± 2°C. Wheat seedlings were grown in a greenhouse under similar conditions. Wheat leaves from 2- to 3-week old seedlings and *C. bonariensis* leaves of 2- or 10-week-old vegetative stages were used in experiments. Seeds of the wheat genotypes were kindly provided by Dr. G.M. Pastori (John Innes Institute, Norwich, UK).

Polyamine Extraction

Free polyamines were extracted according to Flores and Galston (1984) with a slight modification. Fresh leaf samples (250 mg) were ground in liquid nitrogen with acid-washed sand and 1.0 mL of 5% $HClO_4$. The extracts were then vortex stirred for 30 min at 4°C. After centrifugation at 12,000*g* for 5 min, the supernatants were dansylated. A 200-μL aliquot of each extract was added to 400 μL of saturated Na_2CO_3 and 400 μL of dansyl chloride (5 mg per mL of acetone) at room temperature, and mixed for 10 to 16 h in the dark. Excess dansyl chloride was inactivated by adding 100 μL of 10% DL-Pro. Dansylated polyamines were extracted into 0.5 mL of toluene after 1 min of vortex stirring. Phases were separated by centrifugation at 12,000*g* for 3 min. The upper organic phase was collected and immediately used for TLC or stored at −20°C.

Conjugated polyamines were also isolated by the method of Flores and Galston (1984). The $HClO_4$-insoluble fraction was resuspended in 1 mL of 6 N HCl and incubated at 110°C for 24 to 48 h. The hydrolysate was dried at 110°C and resuspended in 5% $HClO_4$. After centrifugation at 12,000*g* for 3 min, the supernatants were collected for dansylation and for toluene extraction, as described above.

Polyamine Analysis

TLC separation was performed on Kieselgel-60 plates (Merck, Darmstadt, Germany) with a concentrating zone. The TLC plates were developed with *n*-hexane:ethyl acetate (5:4, v/v) for 90 min in the dark. The plates were immediately photographed with transmitted UV light. Individual dansylated polyamine bands were identified by comparing the R_F values of dansylated putrescine, spermidine, and spermine standards (Sigma). The dansylated polyamine bands were scraped off the plates and eluted into 2 mL of ethylacetate. After mixing on a stirrer for 2 min and centrifugation at 12,000*g* for 5 min at 4°C, the levels of the polyamines were quantified with a spectrophotofluorimeter (LS-5B, Perkin-Elmer) using an excitation of 350 nm and a measuring emission of 495 nm. The concentration of each polyamine was determined by fluorescence intensity, according to the standard curves generated with polyamine standards treated under the same conditions as the extracts.

Enzyme Assays

Arg and Orn decarboxylase activities of leaf extracts were measured as the release of $^{14}CO_2$ from L-[U-^{14}C]Arg (320.5 mCi/mmol, NEN, kindly provided by Prof. A. Altman [Hebrew University Faculty of Agriculture, Rehovot, Israel]) and DL-[1-^{14}C]Orn (55 mCi/mmol, ARC-200, kindly provided by Prof. C. Kahana [Weizmann Institute of Science, Rehovot, Israel]), respectively, according to methods of Altman et al. (1982) and Tobias and Kahana (1993). The fresh leaves (500 mg) were ground in liquid nitrogen with 2 mL of extraction buffer containing 0.1 M potassium phosphate (pH 7.5), 10 mM DTT, 20 mM sodium ascorbate, 10 mM EDTA, and 1.0 mM pyridoxal phosphate. Purified, insoluble PVP (50 mg/sample) and acid-washed sand (100 mg/sample) were added to the mortar and pestle for extraction. After centrifugation for 20 min at 12,000*g* at 4°C, the supernatants were used for enzyme assay.

The assay mixtures for Arg and Orn decarboxylase activities contained 50 μg of crude extract protein, 0.125 μCi of L-[U-^{14}C]Arg, or 0.1 μCi of DL-[1-^{14}C]Orn, respectively, with extraction buffer to a final reaction volume of 100 μL. Incubation was performed in a 96-well microtiter dish at 37°C for 4 h. Enzyme-liberated $^{14}CO_2$ was trapped on a sheet of Whatman 3MM filter paper impregnated with saturated barium hydroxide solution that was placed under the lid of the microtiter dish, above the wells. At the end of the incubation, the filter paper was washed with 100% acetone for 2 min, dried in air, and then exposed to a phosphor imaging screen (Fuji Photo Film Co., Tokyo, Japan) and scanned. The blank control values were obtained by using boiled crude enzyme. The radioactive spots were cut out from the 3MM filter paper, and the radioactivity was determined in a liquid-scintillation counter (TRI-CARB 1500, Packard Instruments, Downers Grove, IL). The data were then corrected for the differences in specific activities, isotope position, and racemic ratios in the substrates, before transforming to the relative activity values so that Arg and Orn decarboxylase activities would be on a relative molar basis.

Measurement of Oxidant Stress Resistance as Decreased Photosynthesis

Resistance to paraquat (I_{50}) was used as an indicator of oxidant stress resistance in this study. Commercial paraquat (Zeneca, Fernhurst, Surrey, UK) was formulated with 0.2% Tween 20.

Measurements of photosynthetic $^{14}CO_2$ fixation were according to Amsellem et al. (1993) with slight modifications. Detached leaves of *C. bonariensis* and wheat were placed in different concentrations of paraquat for 30 s. Leaves then were placed on wet Whatman 3MM paper in a sealed chamber with illumination of 80 μmol m^{-2} s^{-1} PPFD for 1 h of recovery for wheat leaves and 3 h of recovery for *C. bonariensis*. Then, about 5 μCi of $NaH^{14}CO_3$ (53 mCi/mmol) was put in the sealed chamber, and tartaric acid was remotely dripped on the bicarbonate to release $^{14}CO_2$ and illuminated for another 25 min. At the end of this period, 7-mm-diameter *C. bonariensis* leaf discs or 4-mm-diameter wheat leaf discs were removed from each leaf for measurements on an equal area basis, with three replicates per treatment. The discs were put in 2 mL of 90% acetone in small scintillation vials, and placed under strong light for 48 h to photobleach pigments and evaporate the acetone. A water-miscible scintillation fluid was added to the cleared leaves, and the remaining water and samples were counted as above. The I_{50} values for the resistant and the sensitive plants were calculated from semilogarithmic plots of the dose-response curves.

Paraquat Pretreatment

Two-week-old *C. bonariensis* plants were sprayed with 0.25 mL/cm^2 of 1 μM paraquat in 0.2% Tween 20. Leaves were collected 24 h later for experiments. Leaves from 10-week-old plants were cut 2 and 24 h after 10 μM paraquat pretreatment.

Putrescine Treatment

Three-week-old *C. bonariensis* plants were sprayed with 0.25 mL/cm^2 of different concentrations of putrescine (dissolved in 0.2% Tween 20). The control plants were sprayed with 0.2% Tween 20. After pretreatment, the plants were illuminated at 100 μmol m^{-2} s^{-1} PPFD at 25 ± 2°C for 24 h. The detached leaves of the sensitive and resistant plants were then put in 100 μM paraquat for 30 s as a challenge. The induced levels of oxidant stress resistance were determined by measuring photosynthesis 3 h later, as described above. Plants were pretreated with 100 μM putrescine for 24 h in experiments in which the dose of paraquat was varied.

Protein Assay

Soluble proteins in the centrifuged crude extracts were measured by the method of Bradford (1976) with BSA as the standard.

RESULTS

Polyamines in *C. bonariensis*

We extracted free polyamines from 2- and 10-week-old vegetative *C. bonariensis* plants to ascertain whether there was a correlation with oxidant resistance. At these ages, the I_{50} of the resistant biotype is typically 15- to 30-fold and 50- to 100-fold greater than that of the susceptible biotype, respectively (Amsellem et al., 1993; Ye and Gressel, 1994). The free putrescine levels of the resistant plants are always higher than those in the sensitive plants at both stages (Fig. 1A). Oxidant stress from pretreatment of both biotypes with paraquat at both ages did not significantly increase free polyamine levels.

We acid-hydrolyzed the material in the pellets from the previous experiments to determine whether there were also differences among the conjugated polyamines. The hydrolyzed, conjugated polyamines were separated by the same TLC separation system (Fig. 1B). These hydrolysates included large amounts of dansylated amino acids from the

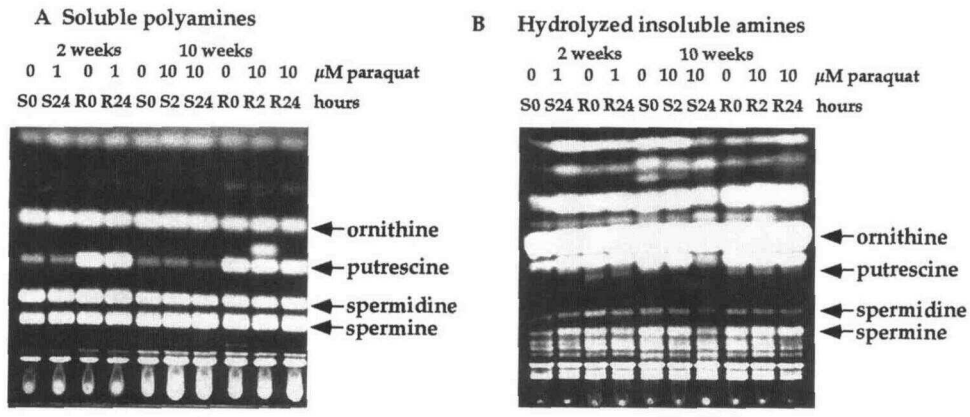

Figure 1. Elevated free (A) and bound (B) putrescine levels in oxidant-stress-resistant (R) and -sensitive (S) *C. bonariensis* leaves. Two- and 10-week-old whole plants were sprayed with 1 or 10 μM paraquat, and the polyamines were directly extracted from detached leaves 0, 2, or 24 h later. The supernatants were used for free polyamine analysis, and the pellet was used for measuring conjugated polyamines after acid hydrolysis. An equal amount of total fluorescence of each dansylated sample was loaded in each lane for TLC. Lanes S0 and R0 are samples of the sensitive and resistant control plants. Lanes S2, S24, R2, and R24 are samples of the sensitive and resistant plants pretreated with paraquat for 2 or 24 h before extraction. For further details, see "Materials and Methods."

Table I. *Correlation of constitutively elevated levels of free putrescine and oxidant stress resistance in 2- and 10-week-old C. bonariensis leaves*

The concentrations of polyamines were determined by scraping from TLC plates (including that shown in Fig. 1A), extracting the polyamines, and measuring their levels by spectrophotofluorimetry, as described in "Materials and Methods." The data are averages ± SE from three separate experiments at 2 weeks, and from four separate experiments at 10 weeks. Note: Different experimental regimes having different recovery times gave quantitatively different magnitudes of resistance in this species; however, the relative resistances were basically the same, with the resistance factor at 10 weeks constitutively much larger than the factor at 2 weeks.

Polyamine/I_{50} Paraquat	Polyamine Levels[a]					
	2 Weeks Old			10 Weeks Old		
	Sensitive	Resistant	R/S[b]	Sensitive	Resistant	R/S[b]
	μmol/g fresh wt					
Putrescine	5.3 ± 1.2	13.3 ± 1.7	2.5[c]	9.5 ± 2	54 ± 6	5.7[d]
Spermidine	24.2 ± 2.3	33.7 ± 15	1.4 (ns)[e]	33 ± 3	61 ± 9.6	1.8[c]
Spermine	187 ± 42	212 ± 44	1.1 (ns)	360 ± 140	365 ± 110	1.0 (ns)
Ratio of (putrescine/spermidine + spermine) × 100	2.3	5.4	2.4[c]	2.4	12.7	5.3[c]
	Oxidant Stress Resistance[f]					
	μM					
I_{50} of paraquat	6	90	15[d]	10	500	50[d]

[a] Corrected for the dilution of the extracts made to give equal total fluorescence before loading on TLC. [b] All of the initial data were analyzed by paired Student's *t* tests before transformation to the ratios of R to S (resistant to sensitive). [c] $P < 0.05$. [d] $P < 0.01$. [e] ns, Not significant ($P > 0.05$). [f] The data for paraquat resistance (I_{50}) at 2 and 10 weeks were from two experiments.

$HClO_4$-precipitated proteins in the pellets. The major polyamine in the conjugates was at the position of Orn, but its identity has not been verified, because there were no differences in levels. The levels of conjugated putrescine (faint band beneath the major Orn band and an unknown band; see Fig. 1B) in the resistant plants were three to four times higher than those in the sensitive biotype. Prestressing with paraquat did not increase the concentration of any of the polyamines in the hydrolysate of the conjugates. This result is consistent with the findings of Bors et al. (1989), who concluded that putrescine is more efficient as a radical scavenger in its conjugated than in its free form.

We then quantified the levels of the different free polyamines from a number of experiments by scraping TLC plates. Relatively high putrescine levels were correlated with oxidant stress resistance in *C. bonariensis* (Table I). The putrescine levels and the ratio of putrescine to other polyamines in 2-week-old-resistant plants were more than double those in the sensitive plants. These higher levels of putrescine in the resistant *C. bonariensis* plants were correlated with the lower level of oxidant-stress resistance at this age, at which the I_{50} of the resistant plants was 15 times higher than that of the sensitive plants. These results are consistent with the data of Szigeti et al. (1996), who showed that paraquat resistance was correlated with a 2.5-fold higher level of putrescine in a paraquat-resistant biotype of *Conyza canadensis*.

There was a further doubling of levels of putrescine and spermidine between the 2- and 10-week stages of the resistant plants. The putrescine levels of the 10-week-old resistant *C. bonariensis* vegetative plants were also measured (Table I). The putrescine levels and the ratio of putrescine to spermidine and spermine in the resistant plants were more than five times higher than in the sensitive plants. The levels of spermidine were almost twice those of the sensitive plants. These results indicate that the constitutively elevated putrescine levels in the resistant plants are correlated with oxidant stress resistance. The I_{50} for paraquat in the resistant plants is >50 times that of the sensitive plants at this time. These results complement the reports of Minton et al. (1990) that paraquat toxicity is increased when putrescine and spermidine synthesis of *Escherichia coli* is defective.

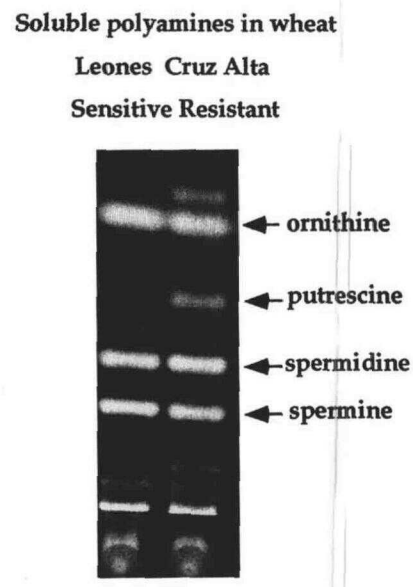

Figure 2. Free putrescine levels are elevated in drought-resistant wheat leaves. Dansylated free polyamines were separated by TLC from resistant and sensitive 2-week-old greenhouse-grown wheat seedlings.

Table II. *Correlation between constitutively elevated levels of free putrescine and oxidant stress resistance in 2-week-old wheat leaves*

The concentrations of polyamines were determined by scraping from TLC plates (including that shown in Fig. 2), extracting the polyamines, and measuring their levels by spectrophotofluorimetry, as described in "Materials and Methods." The data are averages ± SE from four different experiments.

Polyamine/I_{50} Paraquat	Polyamine Levels[a]		
	cv Leones (Sensitive)	cv Cruz Alta (Resistant)	R/S[b]
	μmol/g fresh wt		
Putrescine	14.3 ± 4	40 ± 4	2.8[c]
Spermidine	61 ± 10	76 ± 9.8	1.25[d]
Spermine	300 ± 33	330 ± 16	1.1[e]
Ratio of (putrescine/spermidine + spermine) × 100	4.0	9.9	2.5[d]
	Oxidant Stress Resistance[f]		
	μM		
I_{50} of paraquat	10	100	10[c]

[a] Corrected for the dilution of the extracts made to give equal total fluorescence before loading on TLC. [b] Paired Student's *t* tests were performed on the initial data before transformation to the ratios of R to S (resistant to sensitive). [c] $P < 0.01$. [d] $P < 0.05$. [e] $P > 0.05$. [f] The data for paraquat resistance (I_{50}) at 2 to 3 weeks were from three separate experiments. For further details, see "Materials and Methods."

Polyamines in Wheat

We determined the relative polyamine contents of drought-tolerant and sensitive wheat plants to further ascertain a possible correlation with oxidant tolerance. Higher putrescine levels were constitutively correlated with drought stress tolerance in wheat (Fig. 2). These polyamine patterns in wheat were very similar to those in *C. bonariensis*. Two additional narrow bands and one additional broad band of other amino compounds were also at relatively higher levels in the oxidant-resistant wheat cultivar. The broad band above Orn also correlated with photooxidative tolerance in other wheat cultivars (Müller and Marschner, 1997).

We quantified the actual concentrations of free polyamines, as well as constitutive paraquat resistance (I_{50}) of wheat leaves (Table II). The levels of putrescine in the resistant wheat cv Cruz Alta were nearly 3-fold higher than in the sensitive wheat variety. The level of spermidine was 25% higher in the resistant wheat plants. The ratio of putrescine to spermidine and spermine in the resistant plants was 2.5 times higher than in the sensitive plants. The I_{50} for paraquat in 2-week-old seedlings of the resistant wheat cultivar was 10 times higher than that of the sensitive cultivar. These results are not only consistent with the finding in *C. bonariensis* that constitutively elevated putrescine levels are correlated with oxidant stress resistance, but also further support the idea that drought-stress resistance in wheat is cross-resistant to other oxidative stresses, such as water stress, O_2 stress (Pastori and Trippi, 1993), and paraquat (Table II). Constitutively drought-tolerant biotypes are often cross resistant to paraquat, e.g. in maize (Malan et al., 1990; Pastori and Trippi, 1992) and in other species (Gressel and Galun, 1994). Our results suggest that, at least in some cases, putrescine and other polyamines may be involved in a similar system that protects against oxidative damage from both drought and paraquat stresses.

Arg and Orn Decarboxylase Levels

We assayed enzyme activities in 2- and 10-week-old leaves to determine whether the higher putrescine levels in *C. bonariensis* are a function of an increase in Arg decarboxylase or Orn decarboxylase or both (Table III). The data are corrected for the specific activities, isotope positions, and racemic ratios in the substrates, as described in "Ma-

Table III. *Constitutively elevated Arg (ADC) and Orn decarboxylase (ODC) activities are correlated with paraquat resistance in 2- and 10-week-old C. bonanensis leaves*

Data are averages of three experiments for Arg and Orn decarboxylase activities at 2 and 10 weeks. Relative (R) Arg and Orn decarboxylase activities are compared with the sensitive (S) controls in 2-week-old plants (as 100%). The Arg decarboxylase activity of the leaves of the control plants was 20 pmol h^{-1} mg^{-1} protein. The measurements and calculation of the relative Arg and Orn decarboxylase activities are as described in "Materials and Methods."

Age (Weeks)	ADC			ODC			ADC + ODC		
	S	R	R/S[a]	S	R	R/S[a]	S	R	R/S
2	100	174 ± 20	1.7[b]	ud[d]	ud		100	174	1.7
10	170 ± 30	294 ± 28	1.7[c]	214 ± 11	622 ± 53	2.9[b]	384	916	2.4

[a] Paired Student's *t* tests were performed on the initial data before transformation to the ratios of R to S. [b] $P < 0.05$. [c] $P < 0.01$. [d] ud, Undetectable; the Orn decarboxylase activities at 2 weeks were under the minimum detectable level, which was lower than 10% of the activity that was detected in 10-week-old leaves.

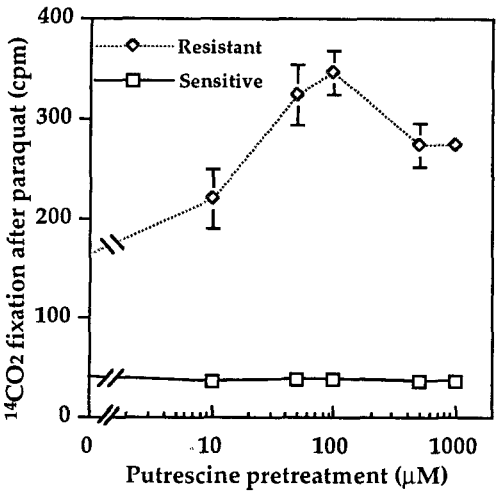

Figure 3. Exogenous putrescine prevents oxidative damage in paraquat-resistant *C. bonariensis*. Three-week-old plants were sprayed with various concentrations of putrescine in 0.2% Tween 20. The detached leaves of control and 24-h putrescine-pretreated plants were challenged with 100 μM paraquat. The $^{14}CO_2$ fixation of control plants without putrescine and paraquat treatments are: sensitive = 198 ± 12 cpm per three 4-mm-diameter leaf discs; resistant = 280 ± 58 cpm in our experimental conditions. SE bars are not shown when they are smaller than the symbols.

terials and Methods." In the 2-week-old-resistant plants, only Arg decarboxylase activities were 70% higher than those in the sensitive plants, and Orn decarboxylase activities of both biotypes were too low to be measured (Table III). These are correlated with the 2.5-times higher level of putrescine in the 2-week-old resistant biotype (Table I).

At 10 weeks, both enzymes were active and the total relative activities were more than twice as high in the resistant plants than in the sensitive biotype (Table III). Arg decarboxylase activities of the resistant plants were almost twice as high as those in the sensitive biotype, and Orn decarboxylase activities were nearly three times those in the sensitive plants. These enzyme activities (Table III) were correlated with the 5.7-fold higher putrescine levels in the resistant biotype at this stage (Table I). Orn decarboxylase activity was double that of Arg decarboxylase in the resistant plants at this time (Table III). These results suggest that both Arg decarboxylase and Orn decarboxylase are involved in synthesizing the putrescine necessary for oxidant stress tolerance, albeit at different stages of development.

Elevated Oxidant Protection by Putrescine

We applied putrescine to 3-week-old *C. bonariensis* leaves to ascertain whether it could enhance their resistance to oxidant stress exerted by paraquat. Both biotypes of *C. bonariensis* plants were sprayed with six concentrations of putrescine. Oxidant stress resistance was measured after another day in light. Putrescine pretreatment completely prevented 100 μM paraquat-induced damage to $^{14}CO_2$ fixation in the resistant biotype, whereas putrescine afforded no protection to the sensitive biotype (Fig. 3). The optimal concentration for induction of oxidant resistance was about 100 μM putrescine (Fig. 3). This dose was used to ascertain the shift in response to different levels of paraquat. The I_{50} of paraquat in putrescine-pretreated resistant plants was double that of the control plants (Fig. 4). There were no significant differences in oxidant resistance in the sensitive biotype between the putrescine-pretreated and control plants.

DISCUSSION

The constitutively elevated antioxidant enzyme activities in 10-week-old paraquat-resistant *Conyza* spp. biotypes are correlated with oxidant-stress resistance (Shaaltiel et al., 1988b; Matsunaka and Ito, 1991; Amsellem et al., 1993; Ye and Gressel, 1994). This resistance is controlled by a single dominant gene (Shaaltiel et al., 1988a). The resistance mechanism is not understood at earlier ages, because the antioxidant enzymes are not significantly higher in the resistant biotype at this time (Amsellem et al., 1993; Norman et al., 1993; Ye and Gressel, 1994). Based on our findings, as well as those of Szigeti et al. (1996), we suggest that putrescine could contribute to the lesser extent of oxidant-stress resistance found at this earlier nonpeak period of resistance in the resistant biotype. The higher levels of putrescine and the high levels of antioxidant enzyme activities in 10-week-old plants could together confer the highest levels of oxidant resistance.

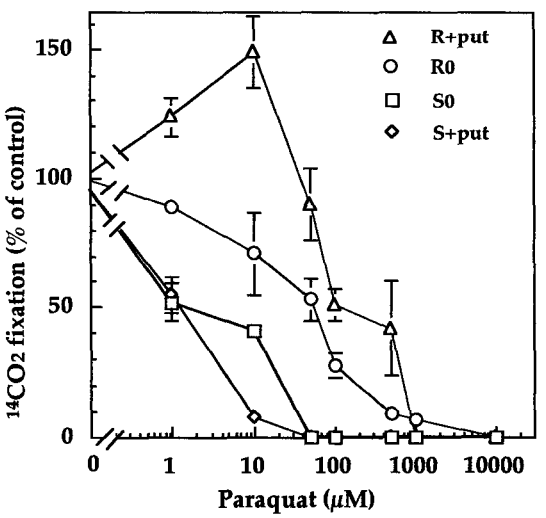

Figure 4. Putrescine application further enhances paraquat resistance in the resistant *C. bonariensis* leaves. Three-week-old sensitive (S) and resistant (R) plants were sprayed with 100 μM putrescine. Leaves were detached and challenged with different concentrations of paraquat 24 h later. S0 and R0 are plants without putrescine pretreatment before paraquat challenge. S+put and R+put are the plants that were pretreated with 100 μM putrescine 24 h before paraquat challenge. The $^{14}CO_2$ fixation of the (100%) controls without paraquat challenge are: S0 = 58 ± 12.6 cpm per three 4-mm-diameter leaf discs; R0 = 155 ± 10 cpm; S+put = 112 ± 15.6 cpm; and R+put = 88 ± 2 cpm. SE bars are not shown when they are smaller than the symbols.

Arg and Orn decarboxylases are key enzymes involved in putrescine biosynthesis (Hiatt et al., 1986). Their relative activities can vary during development (Tiburcio et al., 1988; Evans and Malmberg 1989; Burtin et al., 1991), as was the case with Arg and Orn decarboxylases in *C. bonariensis*. The higher levels of Arg decarboxylase activity in the resistant biotype (Table III) was correlated with the best recovery from paraquat damage (Table I). Arg decarboxylase is located in chloroplasts (Borrell et al., 1995), and they concluded that the role of the polyamine synthesized may be to maintain photosynthetic activity, preventing osmotic-stress-induced senescence. The differences in Arg and Orn decarboxylase activities between 2- and 10-week-old plants suggest that the pathway of putrescine biosynthesis is developmentally regulated. Based on our data so far, we suggest that both the antioxidant enzymes and putrescine might be constitutively linked at the peak period of oxidant tolerance, and are important to obtain the highest levels of oxidant stress resistance.

Putrescine levels were only constitutively elevated in the resistant plants, and the levels were not increased by exogenous oxidative stress in either biotype (Fig. 1), which is similar to the findings in rice, in which putrescine levels are not induced by salt stress (Krishnamurthy and Bhagwat, 1989). Conversely, putrescine biosynthesis was induced by many stresses, such as ozone (Langebartels et al., 1991; Scalet et al., 1995; Tuomainen et al., 1996), wounding (Yoza et al., 1996), osmotic stress (Flores and Galston, 1984), heat shock (Roy and Ghosh, 1996), oxygen deficiency (Reggiani et al., 1990), and K^+ deficiency (Watson and Malmberg, 1996) in other species. Putrescine is known to specifically interact with proteins, nucleic acids, and membranes (Young and Galston, 1983).

Spermidine was also significantly increased in resistant wheat and *C. bonariensis* biotypes, and spermidine is synthesized from putrescine. Thus, the increased spermidine levels may be a function of increased putrescine. Putrescine is also a precursor of γ-aminobutyrate (Flores, 1990), which may function as a signal molecule after stress in plants (Snedden et al., 1995, 1996). Putrescine may be involved in inhibiting paraquat uptake or the transport system, as found by Hart et al. (1992) in an isolated root system.

Because only putrescine levels were greatly enhanced, we only added putrescine exogenously to mimic this phenomenon. Exogenous putrescine application provided direct evidence that putrescine can increase oxidant resistance in *C. bonariensis* (Figs. 3 and 4). This is consistent with the increased oxidant resistance in tomato (Ormrod and Beckerson, 1986) and in tobacco (Bors et al., 1989), in which putrescine, spermidine, and spermine provided considerable protection against ozone injury. The exogenous application of putrescine to the sensitive biotype does not enhance oxidant resistance at the paraquat rates and growth stage used. This may exclude the possibility that putrescine functions as a direct quencher of oxidant in *C. bonariensis*, and may only function in concert with other mechanisms controlled by the single gene conferring resistance. Other polyamines will be tested to ascertain whether they too enhance oxidant tolerance.

The detailed mechanisms of how putrescine and other polyamines confer oxidant-stress resistance still remain unclear (Dumbroff, 1991; Kaur-Sawhney and Galston, 1991). Various possible mechanisms exist that are not mutually exclusive and could be additive:

(a) Putrescine and other polyamines could directly or indirectly function as free radical scavengers (Drolet et al., 1986; Bors et al., 1989).

(b) Putrescine could bind to antioxidant enzymes, such as superoxide dismutase, or be conjugated to small antioxidant molecules and allow them to permeate to the sites of oxidant stress within cells. A covalent putrescine-superoxide dismutase complex was 20-fold more membrane permeable than superoxide dismutase alone, facilitating oxidant protection in a mammalian system (Poduslo and Curran, 1996).

(c) Putrescine and other polyamines might interact with membranes, either by inhibiting transbilayer movement of phospholipids (Bratton, 1994), or by stabilizing molecular complexes of thylakoid membranes (Popovic et al., 1979; Besford et al., 1993).

(d) Endogenous or constitutive putrescine levels might affect the redox state of plant cells. Nagele et al. (1994) reported that the superoxide dismutase-mimetic copper complex of copper-putrescine-pyridine has many functions in mammalian cells. The complex can dismutate superoxide with high efficiency, and enhance oxidation of glutathione. It can protect against peroxide-induced cell injury.

(e) Polyamines may have effects at the gene level, i.e. increased levels of putrescine might either inhibit DNA methylation, permitting the expression of specific genes (Ruiz-Herrera et al., 1995; Martin-Tanguy et al., 1996), or affect gene expression by altering sequence-specific DNA-protein interactions (Burtin et al., 1991; Panagiotidis et al., 1995), or activate or modulate translocation of protein kinases such as CK2 in signal transduction (Shore et al., 1997).

Although our data do not directly or specifically support any of these possibilities, they do show the correlations between oxidant stress tolerance, Arg and Orn decarboxylases, and putrescine levels.

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LITERATURE CITED

Altman A, Friedman R, Levin N (1982) Arginine and ornithine decarboxylases, the polyamine biosynthetic enzymes of mung bean seedlings. Plant Physiol **69:** 876–879

Amsellem Z, Jansen MAK, Driesenaar ARJ, Gressel J (1993) Developmental variability of photooxidative stress tolerance in paraquat-resistant *Conyza*. Plant Physiol **103:** 1097–1106

Aziz A, Larher F (1996) Changes in polyamine titers associated with the proline response and osmotic adjustment of rape leaf discs submitted to osmotic stresses. Plant Sci **112:** 175–186

Basso LC, Smith TA (1974) Effect of mineral deficiency on amine formation in higher plants. Phytochemistry **13:** 875–883

Besford RT, Richardson CM, Campos JL, Tiburcio AF (1993) Effect of polyamines on stabilization of molecular complexes of thylakoid membranes of osmotically stressed oat leaves. Planta **189:** 201–206

Borrell A, Culiañez-Macià FA, Altabella T, Besford RT, Flores D, Tiburcio AF (1995) Arginine decarboxylase is localized in chloroplasts. Plant Physiol **109:** 771–776

Bors W, Langebartels C, Michel C, Sandermann H Jr (1989) Polyamines as radical scavengers and protectants against ozone damage. Phytochemistry **28:** 1589–1595

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bratton DL (1994) Polyamine inhibition of transbilayer movement of plasma membrane phospholipids in the erythrocyte ghost. J Biol Chem **269:** 22517–22523

Burtin D, Martin-Tanguy J, Tepfer D (1991) α-DL-Difluoromethylornithine, a specific, irreversible inhibitor of putrescine biosynthesis, induces a phenotype in tobacco similar to that ascribed to the root-inducing, left-hand transferred DNA of *Agrobacterium rhizogenes*. Plant Physiol **95:** 461–468

Drolet G, Dumbroff EB, Legg R, Thompson JE (1986) Radical scavenging properties of polyamines. Phytochemistry **25:** 367–371

Dumbroff EB (1991) Mechanisms of polyamine action during plant development. *In* AW Galston, AF Tiburcio, eds, Polyamines as Modulators of Plant Development. Juan March Foundation, Madrid, pp 62–66

Evans PT, Malmberg RL (1989) Do polyamines have roles in plant development? Annu Rev Plant Physiol Plant Mol Biol **40:** 235–269

Flores HE (1990) Polyamines and plant stress. *In* GR Alscher, JR Cumming, eds, Stress Responses in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, New York, pp 217–239

Flores HE, Galston AW (1984) Osmotic stress-induced polyamine accumulation in cereal leaves. Physiological parameters of the response. Plant Physiol **75:** 102–109

Foyer CH, Descourvieres P, Kunert KJ (1994) Protection against oxygen radicals: important defense mechanism studied in transgenic plants. Plant Cell Environ **17:** 507–523

Galston AW, Sawhney RK (1990) Polyamines in plant physiology. Plant Physiol **94:** 406–410

Gressel J, Galun E (1994) Genetic controls of photooxidative tolerance. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, FL, pp 237–274

Hart JJ, DiTomaso JM, Linscott DL, Kochian LV (1992) Transport interactions between paraquat and polyamines in roots of intact maize seedlings. Plant Physiol **99:** 1400–1405

Hiatt AC, McIndoo J, Malmberg RL (1986) Regulation of polyamine biosynthesis in tobacco: effects of inhibitors and exogenous polyamines on arginine decarboxylase, ornithine decarboxylase, and *S*-adenosylmethionine decarboxylase. J Biol Chem **261:** 1293–1298

Kaur-Sawhney R, Galston AW (1991) Physiological and biochemical studies on the anti-senescence properties of polyamine in plants. *In* RD Slocum, HE Flores, eds, Biochemistry and Physiology of Polyamines in Plants. CRC Press, Boca Raton, FL, pp 201–211

Kramer GF, Norman HA, Krizek DT, Mirecki RM (1991) Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipid in cucumber. Phytochemistry **30:** 2101–2108

Krishnamurthy R, Bhagwat KA (1989) Polyamines as modulators of salt tolerance in rice cultivars. Plant Physiol **91:** 500–504

Langebartels C, Kerner K, Leonardi S, Schraudner M, Trost M, Heller W, Sandermann H Jr (1991) Biochemical plant responses to ozone. Differential induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol **95:** 882–889

Malan C, Greyling MM, Gressel J (1990) Correlation between CuZn superoxide dismutase and glutathione reductase, and environmental and xenobiotic stress tolerance in maize inbreds. Plant Sci **69:** 157–166

Martin-Tanguy J, Sun L-Y, Burtin D, Vernoy R, Rossin N, Tepfer D (1996) Attenuation of the phenotype caused by the root-inducing, left-hand, transferred DNA and its *rolA* gene. Correlation with changes in polyamine metabolism and DNA methylation. Plant Physiol **111:** 259–267

Matsunaka S, Ito K (1991) Paraquat resistance in Japan. *In* JC Caseley, GW Cussans, RK Atkin, eds, Herbicide Resistance in Weeds and Crops. Butterworth Press, Oxford, UK, pp 77–86

Minton KW, Tabor H, Tabor CW (1990) Paraquat toxicity is increased in *Escherichia coli* defective in synthesis of polyamines. Proc Natl Acad Sci USA **87:** 2851–2855

Müller HH, Marschner H (1997) Use of an in vitro assay to investigate the antioxidative defence potential of wheat genotypes under drought stress as influenced by nitrogen nutrition. Phyton (Horn) (in press)

Nagele A, Felix K, Lengfelder E (1994) Induction of oxidative stress and protection against hydrogen peroxide-mediated cytotoxicity by the superoxide dismutase-mimetic complex copper-putrescine-pyridine. Biochem Pharmacol **47:** 555–562

Norman MA, Fuerst EP, Smeda RJ, Vaughn KC (1993) Evaluation of paraquat resistance mechanisms in *Conyza*. Pestic Biochem Physiol **46:** 236–249

Ormrod DP, Beckerson DW (1986) Polyamines as antiozonants for tomato. HortScience **21:** 1070–1071

Panagiotidis CA, Artandi S, Calame K, Silverstein SJ (1995) Polyamines alter sequence-specific DNA-protein interactions. Nucleic Acids Res **23:** 1800–1809

Pastori GM, Trippi VS (1992) Oxidative stress induces high rate of glutathione reductase synthesis in a drought-resistant maize strain. Plant Cell Physiol **33:** 957–961

Pastori GM, Trippi VS (1993) Cross resistance between water and oxidant stresses in wheat leaves. J Agric Sci **120:** 289–294

Poduslo JF, Curran GL (1996) Increased permeability of superoxide dismutase at the blood-nerve and blood-brain barriers with retained enzymatic activity after covalent modification with naturally occurring polyamine, putrescine. J Neurochem **67:** 734–741

Popovic RB, Kyle DJ, Cohen AS, Zalik S (1979) Stabilization of thylakoid membranes by spermine during stress-induced senescence of barley leaf discs. Plant Physiol **64:** 721–726

Preibe A, Klein H, Jager H-J (1978) Role of polyamines in SO_2-polluted pea plants. J Exp Bot **26:** 1045–1050

Reggiani R, Giussani P, Bertani A (1990) Relationship between the accumulation of putrescine and the tolerance to oxygen-deficit stress in Gramineae seedlings. Plant Cell Physiol **31:** 489–494

Roy M, Ghosh B (1996) Polyamines, both common and uncommon, under heat stress in rice (*Oryza sativa*) callus. Physiol Plant **98:** 196–200

Ruiz-Herrera J, Ruiz-Medrano R, Domínguez A (1995) Selective inhibition of cytosine-DNA methylases by polyamines. FEBS Lett **357:** 192–196

Scalet M, Federico R, Guido MC, Manes F (1995) Peroxidase activity and polyamine changes in response to ozone and simulated acid rain in Aleppo pine needles. Environ Exp Bot **35:** 417–425

Shaaltial Y, Chua N-H, Gepstein S, Gressel J (1988a) Dominant pleiotropy controls enzymes co-segregating with paraquat resistance in *Conyza bonariensis*. Theor Appl Genet **75:** 850–856

Shaaltial Y, Glazer A, Bocion PF, Gressel J (1988b) Cross tolerance to herbicidal and environmental oxidants of plant biotypes to paraquat, sulfur dioxide, and ozone. Pestic Biochem Physiol **31:** 13–23

Shore LJ, Soler AP, Gilmour SK (1997) Ornithine decarboxylase expression leads to translocation and activation of protein kinase CK2 *in vivo*. J Biol Chem **272:** 12536–12543

Snedden WA, Arazi T, Fromm H, Shelp BJ (1995) Calcium/calmodulin activation of soybean glutamate decarboxylase. Plant Physiol **108:** 543–549

Snedden WA, Koutsia N, Baum G, Fromm H (1996) Activation of a recombinant petunia glutamate decarboxylase by calcium/calmodulin or by a monoclonal antibody which recognizes the calmodulin binding domain. J Biol Chem **271:** 4148–4153

Szigeti Z, Rácz I, Darkó E, Lásztity D, Lehoczki E (1996) Are either SOD and catalase or the polyamines involved in the paraquat resistance of *Conyza canadensis*? J Environ Sci Health **31:** 599–604

Tiburcio AF, Kaur-Sawhney R, Galston AW (1988) Polyamine biosynthesis during vegetative and bud differentiation in thin layer tobacco tissue cultures. Plant Cell Physiol **29:** 1241–1249

Tiburcio AF, Masdéu MA, Dumortier FM, Galston AW (1986) Polyamine metabolism and osmotic stress. 1. Relation to protoplast viability. Plant Physiol **82:** 369–374

Tobias KE, Kahana C (1993) Intersubunit location of the active site of mammalian ornithine decarboxylase as determined by hybridization of site-directed mutants. Biochemistry **32:** 5842–5847

Tuomainen J, Pellinen R, Roy S, Kiiskinen M, Eloranta T, Karjalainen R (1996) Ozone affects birch (*Betula pendula* Roth) phenylpropanoid, polyamine and active oxygen detoxifying pathway at biochemical and gene expression level. J Plant Physiol **148:** 179–188

Watson MB, Malmberg RL (1996) Regulation of *Arabidopsis thaliana* (L.) Heynh arginine decarboxylase by potassium deficiency stress. Plant Physiol **111:** 1077–1083

Ye B, Gressel J (1994) Constitutive variation of ascorbate peroxidase activity during development parallels that of superoxide dismutase and glutathione reductase in paraquat-resistant *Conyza*. Plant Sci **102:** 147–151

Young ND, Galston AW (1983) Putrescine and acid stress. Induction of arginine decarboxylase activity and putrescine accumulation by low pH. Plant Physiol **71:** 767–771

Yoza KI, Takeda Y, Sekiya K, Nogata Y, Ohta H (1996) Putrescine accumulation in wounded green banana fruit. Phytochemistry **42:** 331–334